

Different distribution of CD4 and CD8 T cells in synovial membrane and peripheral blood of rheumatoid arthritis and osteoarthritis patients

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Abstract: Rheumatoid arthritis (RA) and osteoarthritis (OA) are chronic diseases associated with morphological joint changes. Synovial membrane (SM) involvement was established for RA, but the data for OA are limited, because OA is usually regarded as noninflammatory disease. Changes in immune system in RA are not limited to joints, and the significant role of T cells of peripheral blood (PB) is not disputable. However, there is still an open debate about PB immunological profile in OA. Therefore, we decided to measure the distribution of CD4⁺ and CD8⁺ T cells, regarding CD28 expression, both in PB and SM of RA and OA patients, on the same day. Altogether, eleven RA patients, 11 OA patients and similar numbers of age-matched healthy controls were included into the study. Flow cytometry was used for T cells subpopulation distinguishing and quantification; monoclonal antibodies against CD3, CD4, CD8 and CD28 with different fluorochromes were used for stainings. The RA patients had significantly higher percentage of CD3⁺CD4⁺ cells in PB as compared to OA patients and relevant control group. Both within the CD4⁺ and CD8⁺ compartments, significantly lower percentages of cells bearing the CD28 marker were found in the PB of OA as compared to RA patients. The proportion of CD3⁺CD4⁺ cells in SM was dependent on age of OA patients, older OA patients had significantly higher value of their SM/blood ratio than RA patients. Older OA subjects were also characterized by higher values of the SM/blood ratio of both CD4⁺CD28⁺ and CD8⁺CD28⁺ subpopulations than RA or younger OA patients. In conclusion, in contrast to the traditional view of OA disease, our results give support to the hypothesis that OA may also (like RA) be a disease with a local immunological involvement.

Keywords: rheumatoid arthritis, osteoarthritis, CD4 T cells, CD8 T cells, CD28, peripheral blood, synovial membrane, immune system

Introduction

Rheumatoid arthritis (RA) as well as osteoarthritis (OA) are associated with joint destruction and mobility impairment. Both diseases are characterized by morphological joint changes including cartilage destruction. Synovial membrane (SM) involvement is significant in RA, but usually limited in OA. As far as RA is concerned, the immunological component both local in joints and systemic is well established, and the significant role of T cells is not disputable. On the other hand OA is mainly regarded as noninflammatory

disease and there are conflicting results of studies focused on immunological mechanism of OA, especially concerning type of immunological cells affecting inflammation in joint tissue. The literature data concerning this issue already suggested that T lymphocytes [1], B lymphocytes [2] or macrophages [3] may play an important role in pathogenesis of OA.

Reports about T cell presence in OA synovium are scarce, usually concentrating on the presence of CD4⁺ or CD8⁺ T cells detected with histochemical methods, and the results are not clear. Some data are available on CD4⁺ and CD8⁺ T cells in the peripheral blood of both groups of patients, but usually the authors did not assess the same cells in the synovium [4,5]. Considering known functional differences between T cells expressing CD28 or not, their importance for RA pathology [6] and virtual lack of information on the

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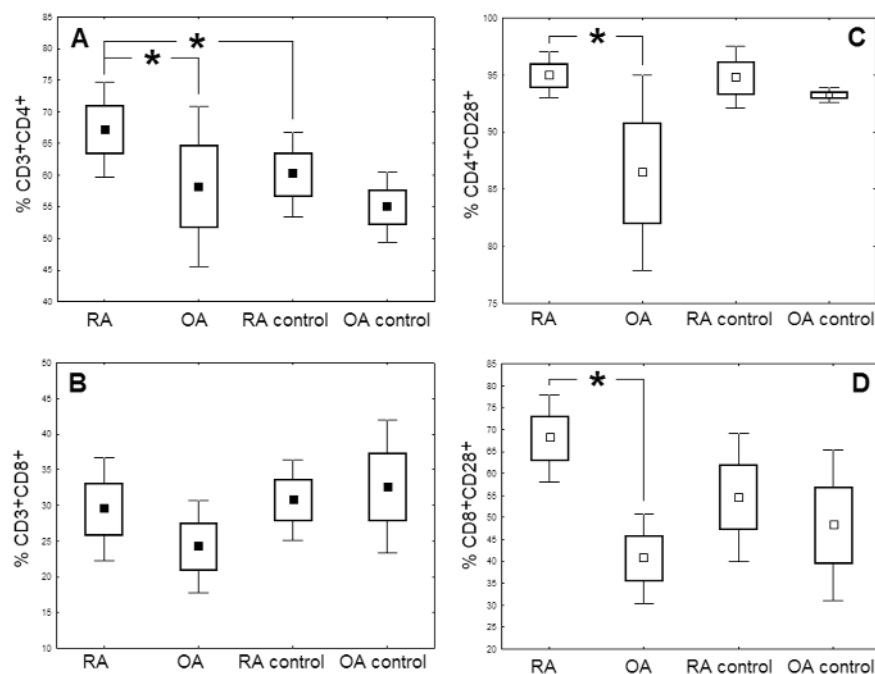


Fig. 1. Proportions of CD3⁺CD4⁺ (A), CD3⁺CD8⁺ (B) lymphocytes and their CD28⁺ subpopulations (C,D) in the peripheral blood of RA and OA patients and relevant controls. Box-and-whisker plots depict means, SD (box) and SEM (whiskers). Asterisks (*) mark statistical significance at $p < 0.05$. Please refer text for further information.

distribution of these two T cell subpopulations in the SM of OA patients, we decided to compare the presence of major T cell populations and their CD28⁺ and CD28⁻ subpopulations in synovium membranes and peripheral blood of OA and RA patients.

Materials and methods

Subjects. Eleven female patients (average age 50.0 ± 7.0 years) with RA and 11 patients with OA (71.0 ± 10.0 years, 4 women and 7 men) were included in the study. Patients were diagnosed and assigned to either RA or OA group by rheumatologist according to the ACR criteria. Due to statistically significant difference in mean age between patient groups, two separate control groups, age- and sex matched to RA and OA patients respectively, were included. The average ages for these groups were: 47.5 ± 5.8 for RA control and 70.6 ± 5.12 for OA control and differed significantly ($p < 0.05$). Participants were informed about the aim of the project and gave their written consent. The project was accepted by the Local Committee for Ethics in Scientific Research at the MUG.

Material. Five ml samples of fasting peripheral venous blood were drawn from each patient and control. Synovial membrane (SM) samples from OA (knee and hip joint) and RA (wrist and hip joint) patients were obtained during therapeutic joint surgery (wrist and knee synovectomy and hip replacement surgery respectively). In order to release cells, synovial tissue was gently cut into small pieces and homogenized by few strokes in a loosely fitting Potter glass homogenizer. The suspension was filtered through a $70 \mu\text{m}$ filter to remove any fragments of solid tissue, then the cells were washed twice with RPMI supplemented with penicillin and streptomycin and the number of viable cells were estimated by light microscope. Prior to staining, the SM cells were suspended in PBS at a concentration approximating that of peripheral blood leukocytes.

Cytometric analysis of peripheral blood and SM T cell subpopulations. One hundred microliters of either peripheral blood or sus-

pended SM cells were stained for 30 minutes on ice with $1 \mu\text{L/mL}$ fluorochrome-conjugated antibodies to CD3, CD4, CD8 (all three from DAKO Cytomation, Denmark), and to CD28 (from Becton Dickinson, USA), or with appropriate isotype control. Red blood cells in the blood samples were lysed by cell lysis buffer for 15 minute at room temperature. Samples were washed twice with PBS to avoid false double positive staining of CD4 and CD8 [7] and then assayed using the FACScan flow cytometer (BD). Ten thousand cells were acquired for each sample. Off-line analysis of the results was performed with the WinMDI™ 2.9 software. Lymphocytes were identified from other cells by forward and side scattered gating, followed by CD3⁺ gating and only the gated cells were analyzed.

Statistical analysis. Statistical analyses were performed with the Statsoft Statistica data analysis software system version 8. P-values ≤ 0.05 were taken to be statistical significance in applied tests. Analysis of variance and Student's t tests was performed to evaluate differences between compared groups and due to small sample and some irregularly distributed variances in some cases appropriate nonparametric tests were applied. P values less than 0.05 were considered as statistical significance and under 0.05 and above 0.10 as borderline statistical significance.

Results

Comparison of percentages of peripheral blood T cell subsets between RA, OA and control groups

The RA patients had significantly higher percentage of CD3⁺CD4⁺ cells as compared to OA patients and relevant control group (Fig. 1A). The proportion of CD3⁺CD8⁺ lymphocytes in the peripheral blood of OA patients was lower than that observed in the blood of RA patients and both control groups, but the differences did not reach statistical significance (Fig. 1B).

Table 1. Proportions of the T cell subpopulations in the synovial membranes of RA and OA patients. The latter were also divided into the 'below 75 years' (OA younger and '75 years and more' (OA older) – explanation in the text.

| Phenotype | RA (a) | OA (b) | OA younger (c) | OA older (d) | Significance |
|--|---------------|---------------|-------------------|-----------------|--------------|
| | % ± SD | % ± SD | % ± SD | % ± SD | p |
| CD3 ⁺ CD4 ⁺ /CD3 ⁺ | 62.06 ± 12.18 | 55.21 ± 22.24 | 47.21 ± 24.39 | 63.19 ± 18.94 | ns |
| CD3 ⁺ CD8 ⁺ /CD3 ⁺ | 50.28 ± 50.49 | 26.57 ± 22.83 | 13.90 ± 9.95 | 39.26 ± 26.25 | ns |
| CD4 ⁺ CD28 ⁺ /CD4 ⁺ | 70.27 ± 17.14 | 61.52 ± 27.27 | 56.96 ± 27.32 | 70.00 ± 29.29 | ns |
| CD8 ⁺ CD28 ⁺ /CD8 ⁺ | 37.70 ± 24.87 | 44.38 ± 24.41 | 39.52 ± 26.59 | 51.67 ± 22.17 | ns |

Both within the CD4⁺ and CD8⁺ compartments, significantly lower percentages of cells bearing the CD28 marker were found in the peripheral blood of OA as compared to RA patients (Fig. 1C,D). Proportion of CD4⁺CD28⁺ lymphocytes was also reduced in the blood of OA patients when compared to the appropriate control group (Fig. 1C).

Despite their significantly different average age, the proportions of T cell subpopulations did not differ significantly between the healthy control groups, although the older (OA control) tended to exhibit lower proportion of CD3⁺CD4⁺, and of CD28⁺ lymphocytes in both CD4⁺ and CD8⁺ populations (Fig. 1).

Calculated values of the CD4/CD8 ratio were not significantly different between patients' groups (not shown).

Comparison of percentages of synovial membrane T cell subsets between RA and OA

We have observed that numbers of lymphocytes in the SM samples, especially obtained from OA patients younger than 75 years were extremely low (not shown). Generally, in these samples lymphocytes accounted for less than 1% of the total cells with the optical features (forward and side scatter) identical with that of lymphocytes (detected in the 'lymphocyte gate' of the FSC/SSC plot), while for older than 75 years the yield of lymphocytes was usually higher. Therefore, for analysis we have divided OA patients into two subgroups: 1) younger than 75 and 2) 75 years and older (average age 65.2 and 79.2 years respectively). Relative proportions of the populations of interest in the SM of RA and OA patients and of the controls are shown in the Table 1.

We did not observe any significant differences between the proportions of studied T cell populations in the SM of RA and OA patients, probably due to extremely high variability of the results. However, OA patients (both as the whole group and after dividing into 'below 75' and '75 and more' age groups tended to have lower proportions of all cell types under study

than the RA individuals, with the notable exception of CD8⁺CD28⁺ cells (Table 1).

Comparison of ratios of the T cell subset percentages in SM to the percentages in peripheral blood (SM/blood ratio) between RA and OA

For further comparison of the proportions of various T cell populations in the SM and blood of RA and OA patients, for each of them we calculated the ratio of the percentages observed for SM cells to the percentages of the peripheral blood lymphocytes of each individual.

With regard to the proportions of CD3⁺CD4⁺ cells, older (75+) OA patients had significantly higher value of their SM/blood ratio than RA patients (Fig. 2A).

As far as SM/blood ratio of CD3⁺CD8⁺ cells is concerned, RA patients and both OA subgroups had comparable ratio values (Fig. 2B).

Older OA subjects were also characterized by higher values of the SM/blood ratio of both CD4⁺CD28⁺ and CD8⁺CD28⁺ subpopulations than either RA (significant differences) or younger OA patients (Fig. 2C, D).

The CD4⁺/CD8⁺ ratio in SM was higher in older OA patients than in RA or younger OA subjects, what was observed with a borderline statistical significance (older OA: 4.2±3.82; RA: 1.52±1.20; younger OA: 1.33±0.62).

Discussion

There is still an open debate about the differences of peripheral blood immunological profile in between OA and RA subjects and literature data in this area are scanty. Leheita *et al.* [8] reported lack of significant differences regarding peripheral blood CD4⁺, CD8⁺ and CD4/CD8 ratio between RA and OA patients.

Our study showed higher percentages of CD3⁺CD4⁺, CD3⁺CD8⁺ and their CD28⁺ subpopulations in RA patients as compared to OA patients and to

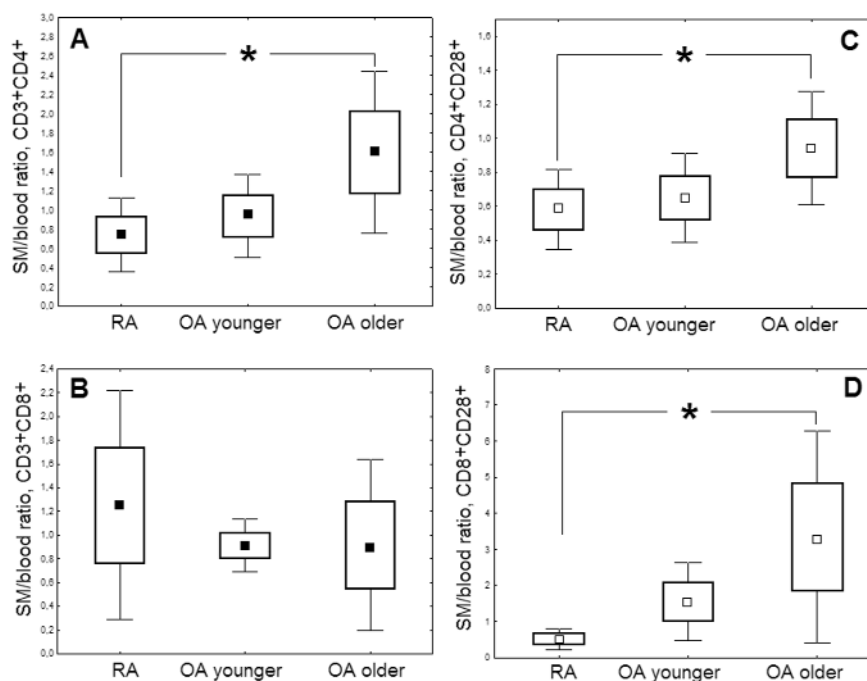


Fig. 2. Ratios of CD3+CD4+ (A), CD3+CD8+ (B) lymphocytes and their CD28+ subpopulations (C,D) in the peripheral blood of RA and OA patients. Box-and-whisker plots depict means, SD (box) and SEM (whiskers). Asterisks (*) mark statistical significance at $p < 0.05$. Please refer text for further information.

age-matched healthy controls. These higher percentages of CD3+CD4+ in peripheral blood of RA patients as compared to control group and OA patients could be a result of increased proliferation of CD4+ T cells due to activated immune response or/and redistribution of CD8+ cells into the synovial membrane, what was observed in those patients.

Another report showed decrease of CD8+ cells both in RA and OA patients when compared to the control groups [4]. These data are in contrast to our results showing comparable percentages of CD3+8+ cells in RA and OA patients as well as in control groups.

We also noticed that RA patients, who underwent joint surgery were significantly younger than OA patients, therefore the interpretation of results should include aging as a significant factor. The proper age of control group seems to be of importance, since the T cell subpopulations change with age, while most studies used the young control group, which can bias the results. Our older controls exhibited a slightly decreased proportion of CD4+ lymphocytes, which is consistent with the observations by Schindowski *et al.* who showed decreased number of CD4+ and CD8+ – positive cells in ageing humans [9]. However, unlike the cited paper, we had not observed any decrease of CD8+ cells in older control group.

Consistent with our study, Hussein *et al.* [5] showed higher CD4/8 ratio in the blood of RA and OA patients compared to the healthy control group; however, in our study the difference did not reach statistical significance, probably due to relatively small examined group.

Published data regarding CD28 expression on CD4 and CD8 T cells of RA patients are also not consistent; the general agreement is that there are fewer CD4+CD28+ and correspondingly more CD4+CD28- cells in the peripheral blood of RA patients compared to the control group; however in other studies the higher frequencies of the latter population were limited to RA patients with extra joints involvement [10].

In the present work we also did not see increased proportion of the CD4+CD28- cells in the blood of RA patients compared to relevant control. Interestingly, we report significant decrease of the proportion of CD4+CD28+ lymphocytes in the peripheral blood of OA patients as compared to the RA ones, corresponding to the reciprocal increase in the CD4+CD28- cells.

However, we found presence of CD4+CD28- cells and decreased CD4+28+ subset in RA patients' synovial membrane, what could suggest preferential migration of the CD4+28- cells in SM in RA patients (compare Table 1 and Fig. 2C).

However, our main task was to compare the distribution of T cells subpopulations in the same patient in peripheral blood and synovial membrane, due to non-consistent results regarding immune system involvement in OA. As a matter of fact, even recent studies describe the phenotypes of immune cells in the RA or OA synovial membranes based on microscopical observations of immunohistochemically stained slides. Thus, one report demonstrated the presence of CD3, CD69, HLA class II, CD25, CD38, CD43, CD45RO cells in the SM obtained from knee and hip joints of

RA and OA patients, showing that there were fewer infiltrating cells in OA than RA patients [1]; the latter seems generally to corroborate our observations shown here. There is no information about percentages, because in that work authors measured only presence and numbers of cells using microscope and high-power field. The authors did not distinguish between CD4 and CD8 T cells [1].

Apparently, SM tissues obtained from various affected joints may differ with respect to immune cell infiltration. Thus, in contrast to analysis mentioned above we did not see lymphocyte infiltration in hip SM obtained from RA patients and only SM obtained from wrist joint was included in our analysis. In our findings percentage of the CD3⁺CD4⁺ cells were higher in SM of OA patients than those of RA subjects what stresses differences in immunological profile in SM between patients groups.

Intensity of T-cell infiltration in the synovia may depend on the local levels of cytokines [1]. Hyc *et al.* demonstrated that pathogen-free rats synovium in vitro and after LPS stimulation produced IL-1 and IL-6 [11]. These proinflammatory cytokines may affect T-cells and be partially responsible for their migration into the inflamed SM. Bondeson *et al.* in a model of culture of synovial cells from OA patients showed that removal of macrophages downregulated production of proinflammatory cytokines and MMPs by synovial fibroblasts, what suggest the pivotal role of macrophages in inflammatory process in OA patients' SM [3].

Also Kennedy *et al.* compared immunological profile of SM obtained from hip and knee joints of RA and OA patients by histological techniques. The study included only 4 patients with OA and 11 patients with RA, but in 3/4 cases of OA and all (11/11) from RA, the synovia contained of the CD4 and CD8 T-lymphocytes, confirming similar occurrence of immune cells' infiltration in the OA synovia [12]. In addition, in the upper synovial region, the differences in proportion of lymphocyte compartments were showed. In the OA patients the numbers of CD4⁺ and CD8⁺ cells were comparable in the synovium, while in the RA synovia there were more CD8⁺ than CD4⁺ cells [12]. Our results obtained with flow cytometric approach are consistent with those cited above, showing similar proportions of CD4⁺ and CD8⁺ cells in the RA synovia, and relatively lower proportion of CD8⁺ lymphocytes in the synovia of OA patients.

Thus, scarce abovementioned works as well as our results confirm the presence of T cells in the SM of both OA and RA patients. The exact role of immunological process in SM in OA is not fully understood. In MRI study, Hill showed that knee synovitis was not associated with cartilage destruction but contributed to knee pain [13]. Miltenburg *et al.* showed that in vitro

activated T-cells from SM of RA and OA patients may have an affect on cartilage destruction by inducing production of collagenases [14].

In addition, in our study we observed decrease percentage of CD4⁺CD28⁺ and CD8⁺CD28⁺ cells in peripheral blood and increase in SM in patients with OA compared with RA patients, what suggest an important role of these cells in OA patients' SM and their migration from periphery to joint tissue. Moreover, the increased presence of CD4⁺ and CD8⁺ with CD28 expression in SM seems to be a feature of older OA patients, aged more than 75 years. It opens an interesting topic about differences between OA pathology in different age, and suggests a possibility that OA in the older patients is more dependent on T cells, which needs to be further examined in the future.

It is important to stress that only few studies so far analyzed fresh SM obtained from patients undergoing joint surgery [3,15]. Their authors prepare SM using DNase and/or collagenase digestion while we adopt a purely mechanical way of releasing the SM cells. It seems that flow cytometry analysis of SM gives more information than conventional immunohistochemical methods, in our opinion due to permitting the analysis of cells infiltrating in a larger volume of tissue that can be feasibly analyzed by microscopy.

In conclusion, in contrast to the traditional view of OA disease, our results give support to the hypothesis that OA may also (like RA) be a disease with a local immunological process. This notion is supported by our finding of the SM/blood percentage ratios for various T cell subpopulations to be different from one, signifying that the lymphocytes present in the synovia are not derived from the contaminating blood. We found that especially in SM obtained from older patients with OA. High values of the SM/blood ratio were observed in these patients regardless of location of disease, duration of the disease and gender. On the other hand, in RA patients the immune cells were only present in SM obtained from wrist joint, regardless of patients' age; this suggests that the joint location sets an important factor for immunological process taking place in SM of RA patients. In addition, we can suggest that CD4⁺CD28⁺ cells are the pivotal cells in SM of RA patients as compared to OA patients, which is in agreement with lowered proportion of those cells present in the peripheral blood of RA patients, supporting the hypothesis of their preferential migration of the CD4⁺CD28⁺ cells into SM of RA and CD4⁺CD28⁺ cells in SM of OA patients.

References

- [1] Sakkas LI, Scanzello C, Johanson N, Burkholder J, Mitra A, Salgame P, Katsetos CD, Platsoucas CD. T cells and T-cell cytokine transcripts in the synovial membrane in patients with osteoarthritis. *Clin Diagn Lab Immunol.* 1998;5(4):430-7.

- [2] Da RR, Qin Y, Baeten D, Zhang Y. B cell clonal expansion and somatic hypermutation of Ig variable heavy chain genes in the synovial membrane of patients with osteoarthritis. *J Immunol.* 2007;178(1):557-65.
- [3] Bondeson J, Wainwright SD, Lauder S, Amos N, Hughes CE. The role of synovial macrophages and macrophage-produced cytokines in driving aggrecanases, matrix metalloproteinases, and other destructive and inflammatory responses in osteoarthritis. *Arthritis Res Ther.* 2006;8(6):R187.
- [4] Kuryliszyn-Moskal A. Comparison of blood and synovial fluid lymphocyte subsets in rheumatoid arthritis and osteoarthritis. *Clin Rheumatol.* 1995;14(1):43-50.
- [5] Hussein MR, Fathi NA, El-Din AM, Hassan HI, Abdullah F, Al-Hakeem E, Backer EA. Alterations of the CD4(+), CD8 (+) T cell subsets, interleukins-1beta, IL-10, IL-17, tumor necrosis factor-alpha and soluble intercellular adhesion molecule-1 in rheumatoid arthritis and osteoarthritis: preliminary observations. *Pathol Oncol Res.* 2008;14(3):321-8.
- [6] Schmidt D, Goronzy JJ, Weyand CM. CD4+ CD7- CD28- T cells are expanded in rheumatoid arthritis and are characterized by autoreactivity. *J Clin Invest.* 1996;97(9):2027-37.
- [7] Bukowska-Straková K, Baran J, Gawlicka M, Kowalczyk D. A false expression of CD8 antigens on CD4+ T cells in a routine flow cytometry analysis. *Folia Histochem Cytobiol.* 2006;44(3):179-83.
- [8] Leheita O, Abed Elrazek NY, Younes S, Mahmoud AZ. Lymphocytes subsets in osteoarthritis versus rheumatoid arthritis. *Egypt J Immunol.* 2005;12(2):113-24.
- [9] Schindowski K, Fröhlich L, Maurer K, Müller WE, Eckert A. Age-related impairment of human T lymphocytes' activation: specific differences between CD4(+) and CD8(+) subsets. *Mech Ageing Dev.* 2002;123(4):375-90.
- [10] Pawlik A, Ostanek L, Brzosko I, Brzosko M, Masiuk M, Machalinski B, Gawronska-Szklarz B. The expansion of CD4+CD28- T cells in patients with rheumatoid arthritis. *Arthritis Res Ther.* 2003;5(4):R210-3.
- [11] Hyc A, Osiecka-Iwan A, Dziunycz P, Moskalewski S. Preparation of rat synovial membrane for studies of cytokine secretion. *Folia Histochem Cytobiol.* 2007;45(1):57-60.
- [12] Kennedy TD, Plater-Zyberk C, Partridge TA, Woodrow DF, Maini RN. Morphometric comparison of synovium from patients with osteoarthritis and rheumatoid arthritis. *J Clin Pathol.* 1988;41(8):847-52.
- [13] Hill CL, Hunter DJ, Niu J, Clancy M, Guermazi A, Genant H, Gale D, Grainger A, Conaghan P, Felson DT. Synovitis detected on magnetic resonance imaging and its relation to pain and cartilage loss in knee osteoarthritis. *Ann Rheum Dis.* 2007;66(12):1599-603.
- [14] Miltenburg AM, Lacraz S, Welgus HG, Dayer JM. Immobilized anti-CD3 antibody activates T cell clones to induce the production of interstitial collagenase, but not tissue inhibitor of metalloproteinases, in monocytic THP-1 cells and dermal fibroblasts. *J Immunol.* 1995;154(6):2655-67.
- [15] Rollín R, Marco F, Jover JA, García-Asenjo JA, Rodríguez L, López-Durán L, Fernández-Gutiérrez B. Early lymphocyte activation in the synovial microenvironment in patients with osteoarthritis: comparison with rheumatoid arthritis patients and healthy controls. *Rheumatol Int.* 2008;28(8):757-64.

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